Attorney Docket Number SY01105K1QKQK

PATENT APPLICATION

NOVEL AVIAN HERPES VIRUS AND USES THEREOF

INVENTORS:

MARK D. COCHRAN, a citizen of the United States, residing at 4506 Horizon Drive, Carlsbad, CA, 92008, U.S.A.

STEPHANIE M. COOK, a citizen of the United States, residing at 4535 Taft Avenue, La Mesa, CA, 91941, U.S.A.

MARTHA A. WILD, a citizen of the United States, residing at 2414 San Marco Avenue, San Diego, CA, 92104, U.S.A.

ASSIGNEE: SCHERING-PLOUGH VETERINARY CORPORATION

PAMELA G. SALKELD
Schering-Plough Corporation
Patent Department, K-6-1, 1990
2000 Galloping Hill Road
Kenilworth, New Jersey 07033-0530
Telephone No.: (908) 298-2135
Facsimile No.: (908) 298-5388

16521_1

ATTORNEY DOCKET NO.: SY01105K1QKQK

NOVEL AVIAN HERPES VIRUS AND USES THEREOF

This application is a continuation-in-part of U.S. Ser. No. 09/426,352, filed Oct. 25, 1999; which is a continuation of U.S. Ser. No. 08/804,372, filed Feb. 21, 1997, now U.S. Patent No. 6,183,753, which is a continuation-in-part of application No. PCT/US95/10245, filed on August 9, 1995, and U.S. Ser. No. 08/663,566, filed on Jun. 13, 1996, now U.S. Patent No. 5,853,733, which is a continuation of U.S. Ser. No. 08/288,065 filed Aug. 9, 1994, now U.S. Patent No. 5,961,982, which is a continuation-in-part of application No. PCT/US93/05681, filed on June 14, 1993 and U.S. Ser. No. 08/023,610, filed on Feb. 26, 1993, now U.S. Patent No. 5,928,648, which is a continuation-in-part of U.S. Ser. No. 07/898,087, filed June 12, 1992. The disclosures of all publications, patents and patent applications are incorporated herein by reference.

Sub B1

Field of the Invention

The present invention relates to recombinant herpesviruses and, more particularly to a novel avian herpesvirus (NAHV) suitable for use as a viral vector for vaccination of birds against disease.

Background of the Invention

The ability to isolate DNA and clone such isolated DNA into bacterial plasmids has greatly expanded the approaches available to make viral vaccines. The methods used to make the present invention involve modifying cloned DNA sequences from various viral pathogens of animals, by insertions, deletions, single or multiple base changes, and subsequent insertions of these modified sequences into the genome of the virus. One utility of the addition of a foreign sequence is achieved when the foreign sequence encodes a foreign protein that is expressed during viral infection of the animal. The resulting live virus may then be used in a vaccine to elicit an immune response in a host animal and provide protection to the animal against disease. A virus with these characteristics is referred to as a viral vector, because it becomes a living vector that will carry and express the foreign protein in the host animal. In effect it becomes an elaborate delivery system for the foreign protein(s).

The application of recombinant DNA techniques to animal viruses in general has a recent history. The first viruses to be engineered have been those with the

smallest genomes. For example, in the case of the papovaviruses, because these viruses are so small and cannot accommodate much extra DNA, their use in genetic engineering has been as defective replicons. Thus, foreign DNA sequence expression from these viruses requires a wild-type helper virus and is limited to cell culture systems. On the other hand, for adenoviruses, there is a small amount of nonessential DNA that can be replaced by foreign sequences limiting its utility as a vector.

Another group of viruses that have been engineered are the poxviruses. One member of this group, vaccinia, has been the subject of much research on foreign gene expression. Poxviruses are large DNA-containing viruses that replicate in the cytoplasm of the infected cell. They have a structure that is unique in that they do not contain any capsid that is based upon icosahedral symmetry or helical symmetry. The poxviruses are most likely to have evolved from bacterial-like microorganisms through the loss of function and degeneration. In part due to this uniqueness, the advances made in the genetic engineering of poxviruses cannot be directly extrapolated to other viral systems, including the avian herpesviruses. The utility of vaccinia as a vaccine vector is in question because of its close relationship to human smallpox and its known pathogenicity to humans. Thus, the use of host-specific avian herpesviruses is a preferred solution to vaccination of poultry. Viral vectoring techniques have been applied to the genomes of several avian herpesviruses (e.g. U.S. Patent No. 6,121,043, U.S. Patent No. 5,965,138, and WO061736A2).

Marek's disease virus (MDV) is the causative agent of Marek's disease, which encompasses fowl paralysis, a common lymphoproliferative disease of chickens. MDV, a naturally occurring herpesvirus, infects bursa-derived and thymus-derived lymphocytes in chickens, and may subsequently induce a lymphoma of thymus-derived lymphocytes. MDV is a designation of a family of avian herpesviruses. For example, MDV (MDV1) is a virulent strain of herpesvirus in chickens, SB-1 (MDV2) is a naturally attenuated herpesvirus strain in chickens, and HVT (MDV3) is a nonpathogenic herpesvirus of turkey.

Since Marek's disease is contagious, the virus has become an important pathogen of chickens, particularly in an environment of large scale breeding such as in the poultry industry. The disease occurs most commonly in young chickens between 2 and 5 months of age. The prominent clinical signs are progressive paralysis of one or more of the extremities, incoordination due to paralysis of legs, drooping of the limb due to wing

involvement, and a lowered head position due to involvement of the neck muscles. In acute cases, severe depression may result. In the case of highly oncogenic strains, there is characteristic bursal and thymic atrophy. In addition, there are lymphoid tumors affecting the gonads, lungs, liver, spleen, kidney and thymus (Mohanty and Dutta, *Veterinary Virology*, Lea and Febiger, pubs., Philadelphia, 1981).

Currently, Marek's disease is controlled by vaccination of embryos at 17-19 days of incubation, or one day old chicks. The principal vaccination method for MDV involves using naturally occurring strains of turkey herpesvirus (HVT) or conventionally attenuated Marek's disease virus (MDV). It would be advantageous to incorporate other antigens into this vaccination, but efforts to combine conventional vaccines have not proven satisfactory due to competition and immunosuppression between pathogens. The multivalent NAHV based vaccines engineered in this invention represent a novel way to simultaneously vaccinate against a number of different pathogens.

A foreign gene of interest targeted for insertion into the genome of NAHV may be obtained from any pathogenic organism of interest. Typically, the gene of interest will be derived from pathogens that in poultry cause diseases that have an economic impact on the poultry industry. The genes may be derived from organisms for which there are existing vaccines, and because of the novel advantages of the vectoring technology, the NAHV derived vaccines will be superior. In addition, the gene of interest may be derived from pathogens for which there is currently no vaccine but where there is a requirement for control of the disease. Typically, the gene of interest encodes immunogenic polypeptides of the pathogen, and may represent surface proteins, secreted proteins and structural proteins.

An avian pathogen that is a target for NAHV vectoring is infectious laryngotracheitis virus (ILTV). ILTV is a member of the herpesviridiae family, and this pathogen causes an acute disease of chickens, which is characterized by respiratory depression, gasping, and expectoration of bloody exudate. Viral replication is limited to cells of the respiratory tract, where in the trachea the infection gives rise to tissue erosion and hemorrhage. In chickens, no drug has been effective in reducing the degree of lesion formation or in decreasing clinical signs. Vaccination of birds with various modified forms of the ILTV derived by cell passage and/or tedious regimes of administration have conferred acceptable protection in susceptible chickens. Because of the degree of attenuation of current ILT vaccines care must be taken to assure that the correct level of

virus is maintained; enough to provide protection, but not enough to cause disease in the flock.

An additional target for the NAHV vectoring approach is Newcastle disease, an infectious, highly contagious and debilitating disease that is caused by the Newcastle disease virus (NDV). NDV is a single-stranded RNA virus of the paramyxovirus family. The various pathotypes of NDV (velogenic, mesogenic, lentogenic) differ with regard to the severity of the disease, the specificity and symptoms, but most types seem to infect the respiratory system and the nervous system. NDV primarily infects chickens, turkeys and other avian species. Historically vaccination has been used to prevent disease, but because of maternal antibody interferences, life-span of the bird and route of administration, the producer needs to adapt immunization protocols to fit specific needs.

Summary of the Invention

The present invention is directed to a recombinant avian herpesvirus comprising a herpes virus of turkeys unique long and repeat viral genome region and a Marek's disease virus unique short viral genome region wherein at least one foreign DNA sequence is inserted within the US2 gene of the unique short region of the recombinant avian herpesvirus and wherein the foreign DNA sequence is capable of being expressed in a host cell. In a preferred embodiment, the foreign DNA sequence is selected from the group consisting of a Newcastle disease virus fusion gene, an infectious laryngotracheitis virus glycoprotein D gene, an infectious laryngotracheitis glycoprotein I gene, or combinations thereof.

In another embodiment, the present invention is directed to a vaccine against Marek's disease, Newcastle disease, and/or infectious laryngotracheitis. The vaccine comprises a recombinant avian herpesvirus comprising a herpes virus of turkeys unique long and repeat viral genome region and a Marek's disease virus unique short viral genome region wherein at least one foreign DNA sequence is inserted within the US2 gene of the unique short region of the recombinant avian herpesvirus and wherein the foreign DNA sequence is capable of being expressed in a host cell, and a suitable carrier. Preferably the foreign DNA sequence is selected from the group consisting of a Newcastle disease virus fusion gene, an infectious laryngotracheitis virus glycoprotein D gene, an infectious laryngotracheitis glycoprotein I gene, or combinations thereof.

The present invention is also directed to a method of immunizing an avian species against Marek's disease, Newcastle disease, and/or infectious laryngotracheitis by administering a vaccine of the present invention.

Brief Description of Figures

Figure 1 is a comparison of HVT, NAHV, and MDV *Bam*HI endonuclease restriction enzyme maps. Restriction fragments are labeled alphabetically in decreasing order of size. The structure of each virus is indicated below the map. Repeats regions are shown as boxes (open = HVT derived, shaded = MDV derived) and the unique regions are shown as lines (single = HVT derived, double = MDV derived). TRL = terminal repeat long; IRL = internal repeat long; IRS = internal repeat short; TRS = terminal repeat short, UL = unique long region; US = unique short region

Figure 2 is a *Bam*HI endonuclease restriction enzyme map of NAHV and the positions of subgenomic clones used in the NAHV construction. Restriction fragments are label alphabetically in decreasing order of size. In the NAHV genome, the fragment corresponding to HVT fragment B is denoted as fragment A' and the fragment corresponding to MDV fragment A is denoted as fragment B'.

Detailed Description of the Invention

The present invention is directed to a recombinant novel avian herpesvirus virus (NAHV) optionally comprising a foreign DNA sequence inserted into a non-essential site in the NAHV genome. The foreign DNA sequence is capable of being expressed in a host cell infected with the recombinant NAHV and its expression is under the control of a promoter located upstream of the foreign DNA sequence. The foreign DNA sequence encodes a polypeptide, which is antigenic in an animal into which the recombinant NAHV is introduced. More particularly, the foreign DNA sequence is from Newcastle disease virus (NDV) or infectious laryngotracheitis virus (ILTV) and the non-essential site in the NAHV genome is the US2 gene.

We have created recombinant organisms consisting of the unique long (UL) and repeat regions of the herpesvirus of turkeys (HVT) and the unique short (US) region of

Marek's disease virus (MDV). The genome structure of these recombinant organisms and their parental viruses are compared in figure 1. Since these organisms are distinctly different from both of their parent organisms, they represent a completely new type of organism, a novel avian herpesvirus (NAHV).

These NAHV provide for highly efficacious and safe vaccines that protect poultry from Marek's disease. They combine the strong protective response provided by antigens from their Marek's disease virus parent with the established safety of their herpesvirus of turkeys parent. The NAHV-based vaccines exhibit increased protection against very virulent strains of MDV relative to HVT-based vaccines. However the NAHV-based vaccines retain the same non-pathogenic non-oncogenic safety profile of HVT.

The NAHV may also be used to create multivalent vaccines against Marek's disease, infectious laryngotracheitis, infectious bursal disease, Newcastle disease, or other poultry diseases. Multivalent viral vaccine strains are created by genetically engineering the NAHV to express antigens from the appropriate disease-causing organism. Several examples of NAHV-based vaccines are described below (examples 1-3).

As defined herein "a non-essential site in the NAHV genome" means a region in the NAHV viral genome, which is not necessary for the viral infection or replication. A "viral genome" or "genomic DNA" means the entire DNA, which the naturally occurring herpesvirus contains. As defined herein, "foreign DNA sequence" or "gene" means any DNA or gene that is exogenous to the genomic DNA. An "open reading frame" is a segment of DNA, which contains codons that can be transcribed into RNA which can be translated into an amino acid sequence and which does not contain a termination codon.

An "immunological composition" of the invention, as used herein, refers to any composition that elicits an immune response in an animal. An immune response is the reaction of the body to foreign substances, without implying a physiologic or pathologic consequence of such a reaction, i.e., without necessarily conferring protective immunity on the animal. An immune response may include one or more of the following: (a) a cell mediated immune response, which involves the production of lymphocytes by the thymus (T cells) in response to exposure to the antigen; and/or (b) a humoral immune response, which involves production of plasma lymphocytes (B cells) in response to antigen exposure with subsequent antibody production. The term "vaccine", as used

herein, broadly refers to any compositions that may be administered to an animal to protect the animal against an infectious disease.

The invention further provides a recombinant NAHV suitable for use as a vaccine against Marek's disease. One example of such a virus is designated NAHV 295-01. This virus is also known as S-HVY-165. The recombinant avian herpesvirus designated NAHV 295-01 is a superior virus vaccine strain against very virulent Marek's disease, in chickens and turkeys, providing the safety of avirulent HVT, with the improved antigenicity of added MDV genes. The NAHV 295-01 recombinant virus vaccine is a superior virus vaccine because a single virus vaccine strain will protect against very virulent MDV. Currently the industry relies on combinations of vaccine strains. Since the NAHV 295-01 virus vaccine strain is genetically defined, it provides superior safety compared to conventional vaccine strains that risk reversion to virulence.

The present invention also provides a recombinant NAHV suitable for use as a vaccine containing a foreign DNA sequence encoding an antigenic polypeptide from NDV. In such case, it is preferred that the antigenic polypeptide is NDV fusion (F) protein. One example of such a virus is designated NAHV/NDV 295-93. This virus is also known as S-HVY-177. The recombinant avian herpesvirus designated NAHV/NDV 295-93 is a multivalent virus vaccine strain against Newcastle disease and very virulent Marek's disease in chickens. It contains a foreign gene encoding the fusion protein of the Newcastle disease virus inserted into the MDV US2 gene of the NAHV.

This recombinant virus vaccine has multiple advantages over conventional vaccines. The NAHV/NDV 295-93 vaccine can be administered *in ovo* without the interference often seen when conventional MDV and NDV vaccines are used. Since the vaccine lacks any NDV virulence genes there is no possibility of reversion to virulence or vaccine induced Newcastle disease. Additionally, the cell-associated nature of the NAHV backbone provides protection from NDV maternal antibody interference. The NAHV/NDV 295-93 recombinant virus vaccine is a superior Marek's disease virus vaccine because a single virus vaccine strain will protect against very virulent MDV. Currently the industry relies on combinations of vaccine strains. Since the NAHV/NDV 295-93 virus vaccine strain is genetically defined, it provides superior safety compared to conventional Marek's vaccine strains that risk reversion to virulence.

The invention further provides recombinant NAHV containing foreign DNA sequence encodes the antigenic polypeptide from an ILTV and encodes ILTV

glycoprotein I and/or ILTV glycoprotein D. One example of such a virus is designated NAHV/ILT 295-149. This virus is also known as S-HVY-176.

The recombinant avian herpesvirus designated NAHV/ILT 295-149 is a multivalent virus vaccine strain against infectious laryngotracheitis and very virulent Marek's disease in chickens. It contains two foreign genes encoding glycoprotein D and glycoprotein I of the infectious laryngotracheitis virus inserted into the MDV US2 gene of the NAHV. This recombinant virus vaccine has multiple advantages over conventional vaccines. The NAHV/ILT 295-149 vaccine can be administered *in ovo* providing increased efficiency. Since the vaccine lacks any ILTV virulence genes there is no possibility of reversion to virulence or vaccine induced laryngotracheitis. The NAHV/ILT 295-149 recombinant virus vaccine is a superior Marek's disease virus vaccine because a single virus vaccine strain will protect against very virulent MDV. Currently the industry relies on combinations of vaccine strains. Since the NAHV/ILT 295-149 virus vaccine strain is genetically defined, it provides superior safety compared to conventional Marek's vaccine strains that risk reversion to virulence.

The novel recombinant avian herpesviruses of the present invention may be used as vaccines or immunological compositions against avian diseases which comprise an effective immunizing amount of a recombinant NAHV of the present invention and a suitable carrier. This invention provides a vaccine useful for immunizing an avian species against Marek's disease, which comprises an effective immunizing amount of the recombinant NAHV, and a suitable carrier.

This invention provides a vaccine useful for immunizing an avian species against Newcastle disease, which comprises an effective immunizing amount of the recombinant NAHV, and a suitable carrier.

This invention provides a vaccine useful for immunizing an avian species against infectious laryngotracheitis, which comprises an effective immunizing amount of the recombinant NAHV, and a suitable carrier.

This invention provides a multivalent vaccine useful for immunizing an avian species against Marek's disease and Newcastle disease, which comprises an effective immunizing amount of the recombinant NAHV and a suitable carrier.

This invention provides a multivalent vaccine useful for immunizing an avian species against Marek's disease and infectious laryngotracheitis, which comprises an effective immunizing amount of the recombinant NAHV and a suitable carrier.

Vaccines of the invention may be combined with other vaccines for other diseases to produce multivalent vaccines. For example, the present invention includes, a multivalent vaccine useful for immunizing an avian species against Marek's disease, Newcastle disease and infectious laryngotracheitis, which comprises a mixture of a first recombinant NAHV, a second recombinant NAHV, and a suitable carrier. One example of such a mixture comprises a first recombinant avian herpesvirus designated NAHV/ILT 295-149, a second recombinant avian herpesvirus designated NAHV/NDV 295-93, and a suitable carrier.

This invention provides an immunological composition which comprises at least one recombinant NAHV and a suitable carrier that elicits an immune response in a host avian species. The immune response can be local or systemic. The immune response can be protective or not be protective.

This invention provide recombinant NAHV, which express foreign DNA, sequences and are useful as vaccines in avian species including but not limited to chickens, turkeys, and ducks. These vaccines may contain either inactivated or live recombinant virus. These vaccines may contain infected cells. The vaccines of the present invention are administered by any of the methods well known to those skilled in the art, for example, by intramuscular, subcutaneous, intraperitoneal, or intravenous injection. The vaccine can be administered *in ovo*. Additional methods for administration of the vaccine well known to those skilled in the art are, for example, intranasally, intraocularly or orally.

For purposes of this invention, the term an "effective immunizing amount" refers to the amount of a substance that is sufficient to produce or elicit an immune response. For the present invention, an "effective immunizing amount" of the recombinant NAHV within the range of 10^2 to 10^9 PFU/dose. In another embodiment the immunizing amount is 10^5 to 10^7 PFU/dose. In a preferred embodiment the immunizing amount is approximately 2000 PFU/dose.

This invention provides methods for vaccination of avian species against disease. The method comprises administering to the animal an effective immunizing dose of the vaccine of the present invention. This invention provides a method for vaccination of an avian species against Marek's disease. It provides a method for vaccination of an avian species Newcastle disease. The present invention provides a method for vaccination of an avian species against infectious laryngotracheitis.

This invention provides methods for the vaccination of an avian species against more than one disease. The diseases can be caused by more than one pathogen. A method is provided for the vaccination of an avian species against Marek's disease and Newcastle disease. The present invention provides a method for vaccination of an avian species against Marek's disease and infectious laryngotracheitis. It also provides a method for vaccination of an avian species against Marek's disease, Newcastle disease and infectious laryngotracheitis.

The term "carrier" refers to a diluent, adjuvant, excipient or vehicle with which a compound is administered. Suitable carriers for the recombinant virus are well known to those skilled in the art and include but are not limited to sterile water, aqueous saline solutions, aqueous dextrose or glycerol solutions, proteins, sugars, etc. One example of such a suitable carrier is a physiologically balanced culture medium containing one or more stabilizing agents such as dimethyl sulfoxide, hydrolyzed proteins, lactose, etc.

This invention is further illustrated in the Methods and Examples sections, which follow. These sections are set forth to aid in an understanding of the invention but is not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter.

Methods

Methods for constructing, selecting and purifying recombinant novel avian herpesviruses are detailed below in the materials, methods and examples. The following serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

Preparation of NAHV Vaccine and Viral Stocks

Novel avian herpesvirus stock samples were prepared by infecting tissue culture cells at a multiplicity of infection of approximately 0.01 PFU/cell in complete F10/199 medium. All incubations were carried out at 39°C in a humidified incubator with 5% CO_2 in air. This medium is composed of a 1:1 mixture of Medium 199 and Nutrient Mixture F10 Ham, 2 mM glutamine, 100 units/ml penicillin, 100 units/ml streptomycin, 1x MEM non-essential amino acids (these components are obtained from BioWhittaker or an equivalent supplier) plus 1% fetal bovine serum. After cytopathic effect was

complete, the medium and cells were harvested and the cells were pelleted at 3000 rpm for 5 minutes in a clinical centrifuge. Infected cells were resuspended in complete medium containing 20% fetal bovine serum, 7% dimethyl sulfoxide (DMSO) and stored frozen at -70°C. For long term storage and shipping virus stocks were stored under liquid nitrogen or in the vapor phase of liquid nitrogen.

Preparation of Poultry Herpesvirus DNA

For the preparation of HVT, MDV and NAHV genomic DNA from the cytoplasm of infected cells, primary chicken embryo fibroblasts were infected at a MOI sufficient to cause extensive cytopathic effect before the cells overgrew. All incubations were carried out at 39°C in a humidified incubator with 5% CO_2 in air. Best DNA yields were obtained by harvesting monolayers maximally infected, but showing incomplete cell lysis (typically 5-7 days). Infected cells were harvested by scraping the cells into the medium using a cell scraper (Costar brand). The cell suspension was centrifuged at 3000 rpm for 10 minutes at 5°C in a GS-3 rotor (Sorvall Instruments). The resultant pellet was resuspended in cold PBS (Dulbecco's Phosphate Buffered Saline, 10 ml/225 cm^2 flask) and subjected to another centrifugation for 10 minutes at 3000 rpm in the cold. After decanting the PBS, the cellular pellet was resuspended in 2 ml/flask of cold RSB buffer (10 mM Tris pH 7.5, 1 mM ethylene diamine tetraacetic acid, disodium salt (EDTA), and 1.5 mM $MgCl_2$). One hundred µl of 20% SDS (5 mM final) was added and mixed by rocking. The sample was poured into a 15 ml conical tube. Tewenty-four µl of Proteinase K was added and mixed by rocking, then incubated at 50°C for ≥ 1 hr. After this period, an equal volume of water-saturated phenol was added to the sample and gently mixed by hand. The sample was spun in a clinical centrifuge for 5 minutes at 3000 rpm to separate the phases. The aqueous layer containing the DNA was transferred to a fresh 15 ml conical tube and the phenol extraction repeated a second time. The aqueous layer containing the DNA was then transferred to a 15 ml corex tube and precipitated by adding one-tenth volume of 3 M sodium acetate (NaAC) and 2.5 volumes of cold 100% ethanol (EtOH). The DNA was pelleted by centrifugation (Sorval, HB4 swinging bucket rotor, 10,000 rpm, 20-30 minutes, 4°C). The DNA pellet was washed with 80% EtOH and centrifuged again for 10 minutes. The pellet was air dried and resuspended in 300 µl TE. All viral DNA was stored at approximately 4°C.

Molecular Biological Techniques

Techniques for the manipulation of bacteria and DNA, including such procedures as digestion with restriction endonucleases, gel electrophoresis, extraction of DNA from gels, ligation, phosphorylation with kinase, treatment with phosphatase, growth of bacterial cultures, transformation of bacteria with DNA, and other molecular biological methods are described by Maniatis *et al* (*Molecular Cloning*, Cold Spring Harbor Laboratory, New York, 1982) and Sambrook *et al* (*Molecular Cloning A Laboratory Manual Second Edition*, Cold Spring Harbor Press, 1989). The polymerase chain reaction (PCR) was used to introduce restriction sites convenient for the manipulation of various DNAs. The procedures used are described by Innis *et al* (*PCR Protocols A Guide to Methods and Applications*, 84-91, Academic Press, Inc., San Diego, 1990). In general, amplified fragments were less than 500 base pairs in size and critical regions of amplified fragments were confirmed by DNA sequencing. Except as noted these techniques were used with minor variation.

DNA Sequencing

DNA sequencing was performed on the Applied Biosystems Automated Sequencer Model 373A (with XL upgrade) per instructions of the manufacturer. Subclones were made to facilitate sequencing. Internal primers were synthesized on an ABI 392 DNA synthesizer or obtained commercially (Genosys Biotechnologies, Inc., The Woodlands, TX). Larger DNA sequences were built utilizing consecutive overlapping primers. Assembly, manipulation and comparison of sequences were performed with DNASTAR programs.

Procedure for Cloning NAHV Subgenomic DNA Fragments

A library of subclones containing overlapping HVT subgenomic fragments was generated as follows. DNA was obtained from the FC-126 strain of HVT (American Type Culture Collection). It was sheared and then size selected on a glycerol gradient as described by van Zijl *et al.*,(*Journal of Virology* **62**, 2191-2195, 1988) with 40-50 kb fragments chosen as the insert population. The pooled fractions were diluted twofold with TE (10 mM Tris pH 7.5, 1 mM EDTA), one-tenth volume of 3M NaAc and 2.5 volumes of ethanol were added, and the DNA was precipitated at 30K rpm in a Beckman SW41 rotor for 1 hr. The sheared fragments were given blunt ends by initial treatment

with T4 DNA polymerase, using low dNTP concentrations to promote 3' overhang removal, followed by treatment with Klenow polymerase to fill in recessed 3' ends. These insert fragments were then ligated to a pWE15 (Strategene) cosmid vector, which had been digested with *Bam*HI, treated with calf intestinal phosphatase, and made blunt by treatment with Klenow polymerase. The ligated mixture was then packaged using Gigapack XL packaging extracts (Stratagene). Ligation and packaging was as recommended by the manufacturer.

Published restriction maps for the enzymes *Bam*HI, *Hin*dIII, and *Xho*I permitted the use of subcloned fragments as specific probes to screen the cosmid library for subclones spanning the genome. Probes were generated from subcloned restriction fragments. The fragments were then labeled using a non-radioactive system (Genius, Boehringer Mannheim). Screening was facilitated by picking colonies into media, followed by growth overnight. Sets of five filters and a master plate were stamped from microtiter dish and again grown overnight. Glycerol was added to the wells to 15% and the plates were frozen at -20°C to provide stock cultures of each colony. Filters were BioRad Colony Lift Membranes and were treated and hybridized per manufacturer's instructions, and washed in 0.1X SSC, 0.1% SDS, 65°C. Positive clones, which hybridized with the non-radioactive probe, were detected according to the Genius kit directions.

Colonies were selected for further analysis on the basis of their hybridization to two or more of the specific probes. These were then digested with *Bam*HI, and compared to published maps of HVT (Buckmaster *et al.*, *J. Gen. Virol.* **69**:2033, 1988). The three cosmids (407-32.2C3, 407-32.1C1, and 407-32.5G6) were obtained in this manner. A detailed description of each clone is given below. It was found that chloramphenicol amplification (Maniatis *et al.*, *Molecular Cloning*, Cold Spring Harbor Laboratory, New York, 1982) was necessary to achieve reasonable yields of DNA from these clones. In addition, one cosmid clone (407-32.5G6) was unstable and had to be grown from the original frozen stock in order to obtain satisfactory DNA preparations.

The pWE15 vector allows the inserts to be excised with *Not*I. However, four *Not*I sites are present in the HVT genome, so that inserts spanning these sites cannot be excised with *Not*I. Two of the *Not*I sites are present in the *Bam*HI B fragment of HVT, this fragment was cloned directly in pSP64 (clone 172-07.BA2). The other two sites are present in the unique short region within the *Bam*HI A fragment. This fragment was

cloned directly in the pWE15 vector. The three sheared cosmids and the two *Bam*HI fragments cover all but a small portion of the ends of the HVT genome. Because these regions are repeated in the internal portions of the genome, all of the genetic information is available.

Marek's Disease Virus (MDV), GA strain, was obtained from the USDA (Agricultural Research Service Regional Poultry Laboratory, East Lansing, MI). In order to clone the short region, a partial *Sma*I digest of the DNA was performed and run out on a 0.6% low melt agarose gel. DNA fragments running greater than 24 kb were chosen as the insert population and excised from the gel. The DNA contained within the gel slice was extracted by using warm phenol, centrifugation, and then the aqueous phase was precipitated with one-tenth volume of 3M NaAc, an equal volume of isopropanol and centrifugation at 30K rpm in a Beckman SW41 rotor for 15 minutes. The pelleted DNA was then rinsed with 80% EtOH, air dried and resuspended in H_20. Since the *Sma*I enzyme leaves blunt ends on the isolated fragments, a blunt end pWE15 cosmid vector was prepared as above. The ligated mixture was then packaged using Gigapack Plus packaging extracts (Stratagene). Ligation and packaging was as recommended by the manufacturer. Colonies were selected for further analysis on the basis of their hybridization to a MDV gD specific probe, and comparison to the published restriction digestion maps.

Procedure for Generating Novel Avian Herpesvirus from Overlapping Subgenomic Fragments

Overlapping subgenomic fragments were cotransfected into chicken embryo fibroblast (CEF) cells by calcium phosphate precipitation, resulting in the regeneration of the NAHV genome. The regeneration was mediated by homologous recombination across the overlapping regions of the fragments. First, cosmid and plasmid DNAs were linearized using an appropriate enzyme, and purified by phenol extraction and ethanol precipitation. Then, approximately one microgram of each linear fragment was combined in H_20 (300 µl final volume) and 37 µl of a 2.5M $CaCl_2$ stock was added. Next, 340 µl of a 2X Hepes buffered saline solution (140 mM NaCl, 1.5 mM Na_2HPO_4, 50 mM HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]), pH 7.05) was added and gently mixed for one minute at room temperature, to allow a fine precipitate to form. The precipitated DNA was added to a subconfluent monolayer of secondary CEF cells, plate

in a 6 cm dish and allowed to absorb to the cells for approximately three hours at 39°C. Media was removed and cells were exposed to a 15% (v/v) Glycerol/PBS solution for one minute. The glycerol solution was removed and the monolayer was washed 3 times with PBS. Monolayers were fed with maintenance media (F10/199, 1% fetal bovine serum (FBS), 2% glutamine, 1% non-essential amino acids (NEAA), 1% penicillin/streptomycin (P/S)), and incubated at 39°C. The next day media was replaced and incubation at 39°C continued. Plaques were visible in three to four days. When cells became overcrowded, infected monolayers were passed up to a larger size dish to allow spread of the virus. For passage, media was aspirated, cells were rinsed twice with PBS, aspirated again, then 0.5 ml trypsin-EDTA was added and incubated for 1-3 minutes at room temperature. Cells were pipetted up and down and diluted to 5 ml with maintenance media. The mixture was transferred to a larger dish and incubated at 39°C. When approximately 70 to 80% cytopathic effect (CPE) was achieved the infected cells were harvested by trypsinizing the monolayer as described above, except the cells were resuspended in F10/M199 Complete Freezing media. Infected cells were placed on ice and DMSO was added to 7%. After 15 minutes on ice, the cells were frozen at -70°C.

Stocks were then plaque purified two times. For each purification, stocks were diluted 1:100,000 in maintenance media and plated on several 10cm dishes of secondary CEFs. After allowing the infected cells to sit down overnight, the infecting media was removed and replaced with 10ml of nutrient agarose (0.8% low melt agarose, Media 199, 1% FBS, 2% glutamine, 1% NEAA 1% P/S), melted and cooled to 42°C, then allowed to harden at room temperature. Plates were then incubated 5 days at 39°C, until plaques formed. Isolated plaques were then picked using a glass Pasteur pipette to make a plug in the agarose. The plug containing the plaque was transfered into 24-well dish of CEFs. The dish was incubated 3 days, then each well was passed to a 6-well dish, and then to a 6 cm dish in 3 more days. Cells were grown until a 50-75% cytopathic effect was seen, then harvested as above and frozen at -70°C. A second purification was the performed as described above to obtain the final stock.

Southern Blot Analysis of Novel Avian Herpesviruses

Total DNA was isolated from a virus stock as described above. One tenth of the resuspended DNA isolated from a flask, 30 µl, was digested in 60 µl volume. Digestions with appropriate restriction endonucleases were performed as directed by the

manufacturer. Digested DNA was loaded into a single well on a 25 cm long 0.7% agarose gel, and run overnight at 45 volts. Gel buffer was 0.5X TBE (a 1:10 dilution of 5X TBE).

Southern blots were performed using Zetaprobe blotting membranes. The alkaline blotting technique for DNA capillary transfer was used exactly as described in the Zetaprobe instruction manual (Section 2.3). The standard hybridization protocol and subsequent washes are also described in the same manual (Section 4.1) except that the membranes are not dried after the final wash. The probe was labeled using the Genius™ non-radioactive DNA labeling and detection kit. Labeling was performed as described in the detection kit instruction manual under section I, "DNA labeling". One half of the labeled material was denatured by boiling, and added to the hybridization buffer. After the hybridization washes described above, the Zetaprobe filters were treated as described in section III, "Immunological Detection", of the Genius™ labeling kit protocol.

Black Plaque Assay for Foreign Gene Expression in Novel Avian Herpesvirus

To analyze expression of foreign antigens expressed by recombinant NAHV viruses, monolayers of CEF cells were infected with recombinant NAHV, overlaid with nutrient agarose media and incubated for 4-5 days at 39°C. Once plaques developed, the agarose overlay was removed from the dish, the monolayer rinsed 1X with PBS, fixed with 100% methanol for 10 minutes at room temperature and the cells air-dried. After re-hydrating the plate with PBS, the primary antibody was diluted to the appropriate dilution with Blotto (5% non-fat milk/50mM Tris pH 7.5/154 mM NaCl) and incubated with the cell monolayer for 2 hours to overnight at room temperature. Unbound antibody was then removed from the cells by washing three times with PBS at room temperature. An alkaline phosphatase conjugated secondary antibody was diluted with Blotto and incubated with the cells for 2 hours at room temperature. Unbound secondary antibody was then removed by washing the cells three times with PBS at room temperature. Next, the monolayer was rinsed in color development buffer (100mM Tris pH 9.5/ 100mM NaCl/ 50mM $MgCl_2$), and then incubated 10 minutes to overnight at room temperature with freshly prepared substrate solution (0.3 mg/ml Nitro Blue tetrazolium + 0.15 mg/ml 5-bromo-4-chloro-3-indolyl phosphatase in color development buffer.) Finally, the

reaction was stopped by replacing the substrate solution with TE (10mM Tris pH 7.5/ 1 mM EDTA). Plaques expressing the correct antigen stained a purplish-black color.

Newcastle Disease Virus (NDV) Fusion Gene cDNA Cloning

cDNA cloning refers to the methods used to convert RNA molecules into DNA molecules. These methods are described in (U. Gubler and B.J Hoffman, *Gene* **25**, 263-269, 1983). These methods may also be accomplished through the use of various commercially available cDNA cloning kits.

For cloning NDV mRNA, primary chicken embryo fibroblast (CEF) cells were infected at 5-10 plaque forming units per cell with the NDV B1 Hitchner strain (American Tissue Type Culture). When cytopathic effect was evident, but before total destruction, the medium was removed and the cells were lysed in 10 ml lysis buffer (4 M guanidine thiocyanate, 0.1% antifoam A, 25 mM sodium citrate pH 7.0, 0.5% N-lauroyl sarcosine, 0.1 M beta-metcaptoethanol). The cell lysate was poured into a sterilized Dounce homogenizer and homogenized on ice 8-10 times until the solution was homogenous. For RNA purification, 8 ml of cell lysate were gently layered over 3.5 ml of CsCl solution (5.7 M CsCl, 25 mM sodium citrate pH 7.0) in Beckman SW41 centrifuge tube. The samples were centrifuged for 18 hrs at 20° C at 36000 rpm in a Beckman SW41 rotor. The tubes were put on ice and the supernatants from the tubes were carefully removed by aspiration to leave the RNA pellet undisturbed. The pellet was resuspended in 400 µl glass distilled water, and 2.6 ml of guanidine solution (7.5 M guanidine-HCL, 25 mM sodium citrate pH 7.0, 5 mM dithiothreitol) were added. The 0.37 volumes of 1 M acetic acid were added, followed by 0.75 volumes of cold ethanol and the sample was put at -20° C for 18 hrs to precipitate RNA. The precipitate was collected by centrifugation in a Sorvall centrifuge for 10 min a 4° C at 10000 rpm in an SS34 rotor. The pellet was dissolved in 1.0 ml distilled water, centrifuged at 13000 rpm, and the supernatant saved. RNA was re-extracted from the pellet 2 more times as above with 0.5 ml distilled water, and the supernatants were pooled. A 0.1 volume of 2 M potassium acetate solution was added to the sample followed by 2 volumes of cold ethanol and the sample was put at -20° C for 18 hrs. The precipitated RNA was collected by centrifugation in the SS34 rotor at 4° C for 10 min at 10000 rpm. The pellet was dissolved in 1 ml distilled water and the concentration taken by absorption at A260/280. The RNA was stored at -70°C.

mRNA containing polyadenylate tails (poly-A) was selected using oligo-dT cellulose (Pharmacia #27 5543-0). Three mg of total RNA was boiled and chilled and applied to the 100 mg oligo-dT cellulose column in binding buffer (0.1 M Tris pH 7.5, 0.5 M LiCl, 5mM EDTA pH 8.0, 0.1% lithium dodecyl sulfate). The retained poly-A RNA was eluted from the column with elution buffer (5mM Tris pH 7.5, 1mM EDTA pH 8.0, 0.1% sodium dodecyl sulfate). This mRNA was reapplied to an oligo-dT column in binding buffer and eluted again in elution buffer. The sample was precipitated with 200 mM sodium acetate and 2 volumes cold ethanol at -20°C for 18 hrs. The RNA was resuspended in 50 µl distilled water.

Ten µg poly-A RNA was denatured in 20 mM methyl mercury hydroxide for 6 min at 22°C. ß-mercaptoethanol was added to 75 mM and the sample was incubated for 5 min at 22°C. The reaction mixture for first strand cDNA synthesis in 0.25 ml contained 1 µg oligo-dT primer (P-L Bio-chemicals) or 1 µg synthetic primer, 28 units placental ribonuclease inhibitor (Bethesda Research Labs #5518SA), 100 mM Tris pH 8.3, 140 mM KCl, 10mM $MgCl_2$, 0.8 mM dATP, dCTP, dGTP, and dTTP (Pharmacia), 100 microcuries ^{32}p-labeled dCTP (New England Nuclear #NEG-013H), and 180 units AMV reverse transcriptase (Molecular Genetics Resources #MG 101). The reaction was incubated at 42°C for 90 min, and then was terminated with 20mM EDTA pH 8.0. The sample was extracted with an equal volume of phenol/chloroform (1:1) and precipitated with 2 M ammonium acetate and 2 volumes of cold ethanol -20°C for 3 hrs. After precipitation and centrifugation, the pellet was dissolved in 100 µl distilled water. The sample was loaded onto a 15 ml G-100 Sephadex column (Pharmacia) in buffer (100 mM Tris pH 7.5, 1 mM EDTA pH 8.0, 100 mM NaCl). The leading edge of the eluted DNA fractions was pooled, and DNA was concentrated by lyophilization until the volume was about 100 µl, then the DNA was precipitated with ammonium acetate plus ethanol as above.

The entire first strand sample was used for second strand reaction which followed the Gubler and Hoffman (1983) method except that 50 µg/ml dNTP's, 5.4 units DNA polymerase I (Boerhinger Mannheim #642-711), and 100 units/ml *E. coli* DNA ligase (New England Biolabs #205) in a total volume of 50 microliters were used. After second strand synthesis, the cDNA was phenol/chloroform extracted and precipitated. The DNA was resuspended in 10 µl distilled water, treated with 1 µg RNase A for 10 min at 22°C, and electrophoresed through a 1% agarose gel (Sigma Type II agarose) in 40 mM Tris-

acetate pH 6.85. The gel was stained with ethidium bromide, and DNA in the expected size range was excised from the gel and electroeluted in 8 mM Tris-acetate pH 6.85. Electroeluted DNA was lyophilized to about 100 microliters, and precipitated with ammonium acetate and ethanol as above. The DNA was resuspended in 20 µl water.

Oligo-dC tails were added to the DNA to facilitate cloning. The reaction contained the DNA, 100 mM potassium cacodylate pH 7.2, 0.2 mM dithiothreitol, 2mM $CaCl_2$, 80 µmoles dCTP, and 25 units terminal deoxynucleotidyl transferase (Molecular Genetic Resources #S1001) in 50 µl. After 30 min at 37°C, the reaction was terminated with 10mM EDTA, and the sample was phenol/chloroform extracted and precipitated as above.

The dC-tailed DNA sample was annealed to 200 ng plasmid vector pBR322 that contained oligo-dG tails (Bethesda Research Labs #5355 SA/SB) in 200 µl of 0.01 M Tris pH 7.5, 0.1 M NaCl, 1 mM EDTA pH 8.0 at 65°C for 2 min and then 57°C for 2 hrs. Fresh competent *E. coli* DH-1 cells were prepared and transformed as described by Hanahan (*Molecular Biology* **166**, 557-580, 1983) using half the annealed cDNA sample in twenty 200 µl aliquots of cells. Transformed cells were plated on L-broth agar plates plus 10 µg/ml tetracycline. Colonies were screened for the presence of inserts into the ampicillin gene using Ampscreen (Bethesda Research Labs #5537 UA), and the positive colonies were picked for analysis. Resulting positive clones were screened for homology to paramyxovirus fusion gene sequences. A clone containing the complete coding sequence of the NDV fusion gene was identified. The sequence of this clone is given in SEQ ID: 1.

Subgenomic Clone 407-32.2C3

Cosmid 407-32.2C3 contains an approximately 40,000 base pair region of genomic HVT DNA (from the left terminus to position 39,750 GenBank Accession No. AF291866, see figure 2). This region includes NAHV *Bam*HI fragments F', L, P, N1, E, D, and 2,092 base pairs of fragment A'. Note: NAHV *Bam*HI fragment A', is called fragment B in HVT. This cosmid may be constructed as described above in the Procedure for Cloning NAHV Subgenomic DNA Fragments. It was isolated from the sheared DNA library by screening with the probes P1 (HVT *Bam*HI fragment F, position 116,948 to 125,961, Genbank Accession No. AF291866) and P2 (HVT *Bam*HI fragment B, 37,663 to 63,593, Genbank Accession No. AF291866). A bacterial strain containing

this cosmid has been deposited pursuant to the Budapest Treaty on the International Deposit of Microorganisms for the Purposes of Patent Procedure with the Patent Culture Depository of the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 U.S.A. under ATCC Accession No. 75430.

Subgenomic Clone 172-07.BA2

Plasmid 172-07.BA2 contains a 25,947 base pair region of genomic HVT DNA. This plasmid was constructed using standard recombinant DNA techniques joining two restriction fragments from the following sources. The first fragment is a 2999 base pair *Bam*HI to *Bam*HI restriction fragment of pSP64 (Promega). The second fragment is the 25,947 base pair *Bam*HI B fragment of HVT (position 37,663 to 63,593, GenBank Accession No. AF291866). Note: In the NAHV genome, this fragment is denoted as fragment A', due to size considerations.

Subgenomic Clone 407-32.5G6

Cosmid 407-32.5G6 contains a 39,404 base pair region of genomic HVT DNA (position 61,852 to 101,255, Genbank Accession No. AF291866). This region includes NAHV *Bam*HI fragments H, C, Q, K1, M, K2, plus 1,742 base pairs of fragment A', and 3,880 base pairs of fragment J. Note: NAHV *Bam*HI fragment A', is called fragment B in HVT. This cosmid was constructed as described above in the Procedure for Cloning NAHV Subgenomic DNA Fragments. It was isolated from the sheared DNA library by screening with the probes P2 (HVT *Bam*HI fragment B, 37,663 to 63,593, Genbank Accession No. AF291866) and P3 (HVT *Bam*HI fragment J, position 97,376 to 102,720, Genbank Accession No. AF291866). A bacterial strain containing this cosmid has been deposited on March 3, 1993 pursuant to the Budapest Treaty on the International Deposit of Microorganisms for the Purposes of Patent Procedure with the Patent Culture Depository of the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 U.S.A. under ATCC Accession No. 75427.

Subgenomic Clone 407-32.1C1

Cosmid 407-32.1C1 contains a 37,444 base pair region of genomic HVT DNA (position 96,095 to 133,538, GenBank Accession No. AF291866, see figure 2). This region includes NAHV *Bam*HI fragments J, G, I, F, O, plus 1,281 base pairs of fragment

K2, and 6,691 base pairs of fragment B'. Note: NAHV *Bam*HI fragment B', is called fragment A in HVT. This cosmid was constructed as described above in the Procedure for Cloning NAHV Subgenomic DNA Fragments. It was isolated from the sheared DNA library by screening with the probes P1 (HVT *Bam*HI fragment F, position 116,948 to 125,961, Genbank Accession No. AF291866) and P4 (4169 base pair *Bgl*II to *Stu*I sub-fragment (position 132,088 to 136,256, GenBank Accession No. AF291866) of HVT *Xho*I fragment #5 (position 128,950 to 136,510, GenBank Accession No. AF291866)). Note: an internal *Stu*I site occurs within the 4169 base pair sub-fragment (position 134,083, GenBank Accession No. AF291866). However this site is methylated and does not cleave in plasmid DNA prepared from standard cloning strains of bacteria. A bacterial strain containing this cosmid has been deposited on March 3, 1993 pursuant to the Budapest Treaty on the International Deposit of Microorganisms for the Purposes of Patent Procedure with the Patent Culture Depository of the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 U.S.A. under ATCC Accession No. 75428.

Subgenomic Clone 989-72.8#1

The cosmid 989-72-8#1 contains the NAHV short region cloned into the cosmid pWE15 (Stratagene). To create a short region cosmid for the NAHV, the US region of MDV was joined with the short repeat regions of HVT by PCR amplification and standard cloning techniques. In this engineered cosmid, the complete MDV US region was used, but the short repeat regions of HVT were shortened to remove the US8 (gE) sequence. This avoids the inclusion of sequence homologous to the US8 gene within the MDV US. Cosmid 989-72.8-1 contains the following DNAs: 9,193 bp of the short repeat region from HVT *Bam*HI B (position 126,848 to 136,040; GenBank Accession No. AF291866), an 8 bp synthetic *Pac*-I linker, 11,156 bp from the MDV US (position 66 to 11,221, GenBank Accession L22174), an 8 bp synthetic *Pac*-I linker, and a second HVT short repeat (position 136,040 to 126,848, GenBank Accession No. AF291866), inverted relative to the other repeat. The pWE15 cosmid vector, used to clone these DNAs, was modified by replacing the 64 bp multiple cloning site (*Eco*RI to *Eco*RI), with a 68 bp synthetic linker (*Eco*RI, I-*Sce*I, *Not*I, *Bam*HI, *Not*I, I-*Sce*I, and *Eco*RI), to allow excision of the insert with the I-*Sce*I enzyme.

Foreign DNA sequences are added into the NAHV genome at a *Kpn*I site with in the MDV US2 gene (position 4646, GenBank Accession L22174). The *Kpn*I site interrupts this 270 amino acid coding region at approximately amino acid 85. Cloning the appropriate foreign DNA sequence into the NAHV short region cosmid, 989-72.8#1 at this KpnI site, creates Subgenomic clones used to introduce foreign genes.

Subgenomic Clone 1002-75.4

The cosmid 1002-75.4 contains a foreign gene encoding the fusion protein of the Newcastle disease virus inserted within the MDV US2 gene of the NAHV short region cosmid, 989-72.8#1. The NDV fusion gene (F) is under the control of the human cytomegalovirus immediate early (HCMV IE) promoter and utilizes the herpes simplex virus thymidine kinase (HSV tk) poly adenylation signal (pA). This cosmid was created using standard DNA cloning techniques. The sequence of the foreign DNA inserted into cosmid 989-72.8#1 is given in SEQ ID 1. This sequence was inserted such that the NDV F and MDV US2 genes are transcribed in the same direction. The source of each region of the insert is indicated in the following table.

Table 1: Source of foreign DNAs inserted into Subgenomic Clone 1002-75.4

Region	Start[a]	End[b]	Source
1	1	36	Synthetic Linker
2	37	1189	HCMV genomic DNA (IE promoter cloned as described in US 5,830,745 and sequenced as described above)
3	1190	1200	Synthetic linker
4	1201	3004	NDV cDNA (F gene cloned and sequenced as described above)
5	3005	3025	Synthetic linker
6	3026	3548	HSV genomic DNA (tk pA position 37,694 to 37,172 GenBank Accession No. D10879)
7	3549	3570	Synthetic linker

[a] Starting position of the region in SEQ. ID: 1

[b] Ending position of the region in SEQ. ID: 1

Subgenomic Clone Vector 1012-89.2

The cosmid 1012-89.2 contains two foreign genes encoding the glycoprotein D and glycoprotein I of the infectious laryngotracheitis virus (ILTV) inserted in to the MDV US2 gene of the NAHV short region cosmid, 989-72.8#1. The ILTV genes are under the control of their endogenous promoters. This cosmid was created using standard DNA cloning techniques. The sequence of the foreign DNA inserted into cosmid 989-72.8#1 is given in SEQ ID 2. This sequence was inserted such that the ILTV gD gene and ILTV gI gene are transcribed in the opposite direction of the MDV US2 genes. The source of each region of the insert is indicated in the following table.

Table 2: Source of foreign DNAs inserted into Subgenomic Clone 1012-89.2

Region	Start[a]	Stop[b]	Source
1	1	18	Synthetic Linker
2	19	3581	ILTV genomic DNA (gD and gI genes (position 10,532 to 14,094 Genbank Accession No. U28832)
3	3582	3605	Synthetic linker

[a] Starting position of the region in SEQ. ID: 2

[b] Ending position of the region in SEQ. ID: 2

Examples

Example 1: The NAHV Designated NAHV 295-01 and the Marek's Disease Recombinant Vaccine (NAHV 295-01)

The NAHV 295-01 recombinant virus was generated according to the Procedure for Generating Novel Avian Herpesvirus from Overlapping Subgenomic Fragments. The following combination of subgenomic clones and enzymes were used: 989-72.8#1 with I-*Sce*I, 407-32.2C3 with *Not*I, 172-07.BA2 with *Bam*HI, 407-32.5G6 with *Not*I, and 407-32.1C1 with *Not*I. (The location of subgenomic clones on the resulting NAHV genome is indicated in figure 2.) The NAHV was shown to have the correct genomic structure using the Southern Blot Analysis of Novel Avian Herpesviruses. Stability of the NAHV 295-01 virus vaccine strain was demonstrated by serial passage 12 times in tissue culture followed by a second Southern blot analysis. This virus strain has been deposited

pursuant to the Budapest Treaty on the International Deposit of Microorganisms for the Purposes of Patent Procedure with the Patent Culture Depository of the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 U.S.A. under ATCC Accession No. _____.

The following studies were conducted to demonstrate the safety of the NAHV 295-01 vaccine, and its effectiveness in protecting against challenge with very virulent Marek's disease virus. In study 1, 18-day-old specific pathogen free (SPF) embryos, or one-day-old chicks were vaccinated with the NAHV 295-01 vaccine. As controls, additional groups of one-day-old chicks were vaccinated with one of two USDA-licensed, conventional vaccines comprised of either HVT or MDV-1/Rispens. Five days post-hatch, vaccinated chicks, and non-vaccinated, control chicks were challenged with virulent MDV/RB1B. Birds were then observed for clinical signs of disease for 7 weeks, then necropsied to examine for gross lesions. The results, in Table 3, show the NAHV 295-01 vaccine gave greater protection against very virulent Marek's disease challenge than either commercial vaccine.

Table 3: Efficacy of the NAHV 295-01 Recombinant Vaccine Against Virulent Marek's Disease Virus Challenge

Group	Route	Dose[a]	Challenge[b]	Protection Ratio[c]	% Protected
Non-vac.	--	--	--	25/25	--
Non-vac.	--	--	RB1B	1/30	3%
NAHV 295-01	*in ovo*	895 pfu	RB1B	28/30	93%
NAHV 295-01	SC	940 pfu	RB1B	29/30	97%
HVT	SC	As per label	RB1B	18/30	60%
Rispens	SC	As per label	RB1B	27/30	90%

a *in ovo* dose: PFU/0.05 ml; SC dose: PFU/0.2 ml
b Challenge 5 days post-vaccination, intra-abdominal
c No. protected/Total on day 54

In the second study, 18-day-old embryos or one-day-old SPF chicks were vaccinated with ten times the maximum dose of the NAHV 295-01 vaccine. The chicks were observed for 120 days for clinical signs of Marek's disease, then necropsied and

examined for Marek's lesions. As controls, a third group of birds remained un-vaccinated, and a fourth group of un-vaccinated birds was challenged on day 4 with virulent MDV/RB1B to demonstrate that the birds were susceptible to Marek's disease. The results, in Table 4, demonstrate the safety of the NAHV 295-01 vaccine given *in ovo* (18-day-old embryos) or at one day-of-age.

Table 4: Safety the NAHV 295-01 Recombinant Vaccine Following *in ovo* or Subcutaneous Injection with 10x Dose.

Group	Route	Dose[a]	Challenge[b]	% MD[c]
Non-vac.	--	--	--	--
Non-vac.	--	--	RB1B	100%
NAHV 295-01	*in ovo*	20,000 pfu	--	0%
NAHV 295-01	SC	20,000 pfu	--	0%

a dose: PFU/0.05 ml (In ovo) or PFU/0.2 ml (SC).
b Challenge 5 days post-vaccination; intra-abdominal.
c Percentage MD positive/Total by day 120.

Example 2: The NAHV designated NAHV/NDV 295-93 and the Multivalent Marek's Disease/Newcastle Disease Recombinant Vaccine (NAHV/NDV 295-93)

The NAHV/NDV 295-93 recombinant virus was generated according to the Procedure for Generating Novel Avian Herpesvirus from Overlapping Subgenomic Fragments. The following combination of subgenomic clones and enzymes were used: 1002-75.4 with I-*Sce*I, 407-32.2C3 with *Not*I, 172-07.BA2 with *Bam*HI, 407-32.5G6 with *Not*I, and 407-32.1C1 with *Not*I. (The location of subgenomic clones on the resulting NAHV genome is indicated in figure 2.) The NAHV was shown to have the correct genomic structure using the Southern Blot Analysis of Novel Avian Herpesviruses. Stability of the NAHV/NDV 295-93 virus vaccine strain was demonstrated by serial passage 12 times in tissue culture followed by a second Southern blot analysis. This virus strain has been deposited pursuant to the Budapest Treaty on the International Deposit of Microorganisms for the Purposes of Patent Procedure with the Patent Culture Depository of the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 U.S.A. under ATCC Accession No. _____.

The Black Plaque Assay for Foreign Gene Expression in Novel Avian Herpesvirus was used to demonstrate expression of the NDV fusion gene. The assay used a monoclonal antibody directed to the NDV fusion gene (3-1G5) diluted 1:100 as the primary antibody. A goat anti-mouse alkaline phosphatase conjugated antibody diluted 1:1000 was used as the secondary antibody. Purity of the virus was demonstrated by assay of serial passage 12 stocks, 97.6% (1029/1054) of the viral plaques were black plaque positive.

The following studies were conducted to demonstrate the effectiveness of the NAHV/NDV 295-93 vaccine, in protecting against challenge with either virulent Newcastle disease virus, or very virulent Marek's disease virus. In Study 1, 18-day-old specific pathogen free (SPF) embryos, or one-day-old chicks were vaccinated with the NAHV/NDV 295-93 vaccine. As a control, a third group of one-day-old chicks remained un-vaccinated. Twenty-one days post-hatch, vaccinated chicks, and non-vaccinated, control chicks were challenged with virulent NDV/Texas GB strain. Birds were then observed for clinical signs of disease for fourteen days. The results, in Table 5, show the NAHV/NDV 295-93 vaccine gave better than 90% protection against virulent NDV.

Table 5: Efficacy of the NAHV/NDV 295-93 Recombinant Vaccine Against Virulent Newcastle Disease Virus Challenge

Group	Route	Dose[a]	Challenge[b]	% Protected[c]
Non-vac.	--	--	--	--
Non-vac.	--	--	Texas GB	0%
NAHV/NDV 295-93	*in ovo*	525 pfu	Texas GB	90%
NAHV/NDV 295-93	SC	707 pfu	Texas GB	97%

a *In ovo* dose: PFU/0.05 ml; SC dose: PFU/0.2 ml.
b Challenge 21 days post-vaccination, intra-ocular.
c Percentage Protected/Total; 14 days post-challenge.

In study two, 18-day-old embryos, or one-day-old SPF chicks were vaccinated with the NAHV/NDV 295-93 vaccine. As controls, additional groups of one-day-old chicks were vaccinated with one of two USDA-licensed, conventional vaccines comprised of either HVT or MDV-1/Rispens. Five days post-hatch, vaccinated chicks, and non-vaccinated, control chicks were challenged with virulent MDV/RB1B. Birds

were observed for clinical signs of disease for 7 weeks, then necropsied to examine for gross lesions. The results, in Table 6, show the NAHV/NDV 295-93 vaccine protected against very virulent Marek's disease challenge.

Table 6: Efficacy of the NAHV/NDV 295-93 Recombinant Vaccine Against Virulent Marek's Disease Virus Challenge

Group	Route	Dose[a]	Challenge[b]	Protection Ratio[c]	% Protected
Non-vac.	--	--	--	25/25	--
Non-vac.	--	--	RB1B	1/30	3%
NAHV/NDV 295-93	*in ovo*	675 pfu	RB1B	28/30	93%
NAHV/NDV 295-93	SC	680 pfu	RB1B	26/30	87%
HVT	SC	As per label	RB1B	18/30	60%
Rispens	SC	As per label	RB1B	27/30	90%

a *In ovo* dose: PFU/0.05 ml; SC dose: PFU/0.2 ml.
b Challenge 5 days post-vaccination, intra-abdominal.
c No. protected/Total on day 54.

Example 3: The NAHV Designated NAHV/ILT 295-149 and the Multivalent Marek's Disease/Infectious Laryngotrachetitis Recombinant Vaccine (NAHV/ILT 295-149)

The NAHV/ILT 295-149 recombinant virus was generated according to the Procedure for Generating Novel Avian Herpesvirus from Overlapping Subgenomic Fragments. The following combination of subgenomic clones and enzymes were used: 1012-89.2 with I-*Sce*I, 407-32.2C3 with *Not*I, 172-07.BA2 with *Bam*HI, 407-32.5G6 with *Not*I, and 407-32.1C1 with *Not*I. (The location of subgenomic clones on the resulting NAHV genome is indicated in figure 2.) The NAHV was shown to have the correct genomic structure using the Southern Blot Analysis of Novel Avian Herpesviruses. Stability of the the NAHV/ILT 295-149 virus vaccine strain was demonstrated by serial passage 12 times in tissue culture followed by a second Southern blot analysis. This virus strain has been deposited pursuant to the Budapest Treaty on the International Deposit of Microorganisms for the Purposes of Patent Procedure with the Patent Culture Depository of the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 U.S.A. under ATCC Accession No. _____.

The Black Plaque Assay for Foreign Gene Expression in Novel Avian Herpesvirus was used to demonstrate expression of the ILT glycoproteins. The assay used a convalescent ILT chicken sera (SPAFAS, Inc.) diluted 1:100 as the primary antibody. A goat anti-chicken alkaline phosphatase conjugated antibody diluted 1:1000 was used as the secondary antibody. Purity of the virus was demonstrated by assay of serial passage 12 stocks. 99.4% (1043/1049) of the viral plaques were black plaque positive.

The following studies were conducted to demonstrate the effectiveness of the NAHV/ILT 295-149 vaccine, in protecting against challenge with either virulent infectious laryngotracheitis virus, or very virulent Marek's disease virus. In study 1, 18-day-old specific pathogen free (SPF) embryos, or one-day-old chicks were vaccinated with the NAHV/ILT 295-149 vaccine. As controls, additional groups of one-day-old chicks either remained un-vaccinated, or were vaccinated with a USDA-licensed, conventional vaccine comprised of attenuated, live ILTV (LT-Ivax). Twenty-five days post-hatch, vaccinated chicks, and non-vaccinated, control chicks were challenged with virulent ILT/USDA LT-96-3. Birds were observed for clinical signs of disease for ten days, and then necropsied to examine for gross lesions. The results, in Table 7, show the NAHV/ILT 295-149 vaccine gave better protection against virulent ILT than the commercial vaccine.

Table 7: Efficacy of the NAHV/ILT 295-149 Recombinant Vaccine Against Virulent Infectious Laryngotracheitis Virus Challenge

Group	Route	Dose[a]	Challenge[b]	% Protected[c]
Non-vac.	--	--	--	--
Non-vac.	--	--	ILT(USDA)	0%
NAHV/ILT 295-149	*in ovo*	750 pfu	ILT(USDA)	100%
NAHV/ILT 295-149	SC	750 pfu	ILT(USDA)	100%
LT-Ivax	Per label	Per label	ILT(USDA)	60%

a *In ovo* dose: PFU/0.05 ml; SC dose: PFU/0.2 ml.
b Challenge 25 days post-vaccination, intra-tracheal.
c Percentage Protected/Total; 10 days post-challenge

In study two, 18-day-old embryos, or one-day-old SPF chicks were vaccinated with NAHV/ILT 295-149. As controls, additional groups of one-day-old chicks were vaccinated with a USDA-licensed, conventional vaccine comprised of MDV-1/Rispens, or left un-vaccinated. Five days post-hatch, vaccinated chicks, and non-vaccinated, control chicks were challenged with virulent MDV/RB1B. Birds were observed for clinical signs of disease for 7 weeks, then necropsied to examine for gross lesions. The results, in Table 8, show the NAHV/ILT 295-149 vaccine protected better against virulent Marek's disease challenge, than the commercial vaccine.

Table 8: Efficacy of the NAHV/ILT 295-149 Recombinant Vaccine Against Virulent Marek's Disease Virus Challenge

Group	Route	Dose[a]	Challenge[b]	Protection Ratio[c]	% Protected
Non-vac.	--	--	--	33/33	--
Non-vac.	--	--	RB1B	2/35	6%
NAHV/ILT 295-149	*in ovo*	1500 pfu	RB1B	25/26	96%
NAHV/ILT 295-149	SC	1500 pfu	RB1B	32/34	94%
Rispens	SC	As per label	RB1B	26/33	79%

a *In ovo* dose: PFU/0.05 ml; SC dose: PFU/0.2 ml.
b Challenge 5 days post-vaccination, intra-abdominal

Although certain presently preferred embodiments of the invention have been disclosed herein, it will be apparent to those skilled in the art to which the invention pertains that variation and modification of described embodiments may be made without departing from the spirit and scope of the invention. Accordingly, it is intended that the invention be limited to the extent required by the appended claims and the applicable rules of law.